



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 1/20, 9/99		A1	(11) International Publication Number: WO 96/29392
		(43) International Publication Date: 26 September 1996 (26.09.96)	
(21) International Application Number: PCT/AU96/00167 (22) International Filing Date: 25 March 1996 (25.03.96) (30) Priority Data: PN 1912 23 March 1995 (23.03.95) AU (71) Applicant (for all designated States except US): UNISEARCH LIMITED [AU/AU]; 221-227 Anzac Parade, Kensington, NSW 2033 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only): KJELLEBERG, Staffan [SE/AU]; 22 Goorawahl Avenue, La Perouse, NSW 2036 (AU). STEINBERG, Peter [US/AU]; 144 Denison Street, Newton, NSW 2042 (AU). DE NYS, Peter, Canisius [NZ/AU]; 3/7 Palmerston Avenue, Bronte, NSW 2024 (AU). MAXIMILIEN, Ria [AU/AU]; 138 Kurrewa Avenue, Coogee, NSW 2034 (AU). MANEFIELD, Michael [AU/AU]; 2/40 Wairoa Avenue, Bondi Beach, NSW 2030 (AU). GIVSKOV, Michael [DK/DK]; Soetedams Dosseringen 59C, DK-2100 Copenhagen (DK). GRAM, Lone [DK/DK]; Ellemosevej 20, DK-2900 Hellerup (DK). (74) Agent: F. B. RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: METHODS FOR MICROBIAL REGULATION			
(57) Abstract			
A method and microbial culture medium for inhibiting homoserine lactone (HSL) and/or acylated homoserine lactose (AHL) regulated processes in microorganisms using furanone compounds.			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

Methods for Microbial Regulation

Technical Field

The present invention relates to methods for regulating processes of microorganisms. In particular, methods and culture media including furanones for inhibiting homoserine lactone (HSL) and/or acylated homoserine lactone (AHL) regulated processes in microorganisms.

Background Art

Microorganisms, including bacteria, fungi and algae, have a profound effect upon the activities of humans and animals. Considerable efforts have been made to find compounds and methods for the control of such micro organisms. There is a need to continually find new compounds and methods for this purpose is due, in part at least, to the capacity of such microorganisms to rapidly mutate to circumvent the compounds and methods already developed.

One set of fundamental regulator agents which are widespread in bacteria, including human pathogens, are known as homoserine lactones (HSL) or acylated homoserine lactones (AHL). The AHL regulatory systems in bacteria are two component regulatory systems which regulate intercellular activity in response to environmental conditions and extracellular signal molecules. This system was first discovered in the bioluminescent marine bacteria *Vibrio harveyi* and *V. fischeri* where it is used to control expression of bioluminescence. In principle, the system is comprised of two proteins - LuxR and LuxI. The LuxI enzyme is encoded by a *luxI* gene and produces a related family of signal molecules known as the homoserine lactones. These signal molecules bind to the LuxR regulator which is then activated and serves both as a positive regulator for the structural genes which encode the enzymes responsible for bioluminescence, and as a positive regulator for the *luxI* gene itself. The entire system is amplified via a process of auto induction. Additional molecules serve as regulators of the LuxR-LuxI system.

While initially discovered for bioluminescent bacteria, this regulatory system has now been found in numerous other microorganisms, and is involved in a wide variety of bacterial activities (Swift et al. 1994, Fuqua et al. 1994). These activities include, but are not restricted to, exoenzyme production in the plant pathogen *Erwinia carotovora* and in *Pseudomonas aeruginosa*, the causative agent of cystic fibrosis, and Ti

plasmid transfer from *Agrobacterium tumefaciens* to plants. In all instances, acylated homoserine lactone, or homoserine lactone-like compounds are the regulatory auto inducers.

Since these regulatory systems are widespread among bacteria, and because they control processes leading to bacterial invasion of host organisms, it is likely that other organisms will have evolved defence mechanisms against these systems. So far none have been found. The present inventors have now developed methods of inhibiting microbial processes using compounds that are mimics of the homoserine lactone family of regulatory compounds.

Disclosure of the Invention

Accordingly, in a first aspect the present invention consists in a method of inhibiting an homoserine lactone (HSL) and/or acylated homoserine lactone (AHL) regulated process in a microorganism comprising exposing the microorganism to a furanone compound so as to inhibit the process.

In a preferred form, the furanone compound is derived from the alga *Delisea pulchra* or chemical modifications or derivatives thereof. More preferably the furanone compound is selected from the group consisting of the compounds of Formula 1 and Formula 2 and even more preferably, the furanone compound is selected from the group consisting of the compounds of Figure 3.

The furanone compounds are typically used at a concentration greater than 10 ng/ml. Preferably the furanone compounds are used at concentration from 100 ng to 1 mg/ml and more preferably used at a concentration from 1 µg to 100 µg/ml.

The HSL and AHL regulated processes that can be inhibited by the present invention include motility, swarming, swimming, exoenzyme production, indigo formation, adhesion, attachment and luminescence. It will be appreciated by persons skilled in the art, however, that other AHL and/or HSL regulated processes could also be inhibited by the methods of the present invention.

In order to inhibit the motility or swarming of microorganisms when cultured *in vitro*, the furanones used in the present invention can be included in culture media so that when the motile microorganisms are grown in or on the media their motility or swarming processes are inhibited. This

has particular utility when mixed cultures of microorganisms are cultured on semi-solid agar culture plates where there is a problem of one motile microorganism swarming over the plate and preventing the isolation or selection of other non-motile microorganisms present in the mixture. The incorporation of one or more of the furanones in a culture medium would inhibit the motility or swarming processes regulated by HSL and/or AHL of any microorganisms cultured thereon.

The method of the present invention is applicable for inhibiting processes in microbial pathogens of plants, animals and humans *in vivo*. Typical plant pathogens include *Serratia liquiadiens* and *Erwinia carotovora*, animal pathogens include *Vibrio harveyi* and *Vibrio anguillarum* and human pathogens include *Pseudomonas aeruginosa* and *Proteus mirabilis*.

It will be appreciated that the methods of the present invention can be carried out on microorganisms by treating them directly with furanones as well as indirectly by, for example, giving or applying furanones to plants, animals or humans so as to inhibit HSL and/or AHL regulated processes of the microorganisms of interest in or on the plants, animals or humans. The effect of the furanones will be the reduction or inhibition of the regulated process of interest, irrespective of specific action on the HSL and/or AHL pathway itself.

In a second aspect, the present invention consists in a microbial culture medium for inhibiting an homoserine lactone (HSL) and/or acylated homoserine lactone (AHL) regulated process in a microorganism, the medium comprising growth and/or maintenance ingredients and a furanone compound.

In a preferred form, the furanone compound is derived from the alga *Delisea pulchra* or chemical modifications or derivatives thereof. More preferably the furanone compound is selected from the group consisting of the compounds of Formula 1 and Formula 2 and even more preferably, the furanone compound is selected from the group consisting of the compounds of Figure 3.

The furanone compound is typically included in the medium to give a final concentration in use of greater than 10 ng/ml. Preferably the furanone compound has a final concentration from 100 ng to 1 mg/ml and more preferably the furanone compound has a final concentration from 1 µg to 100 µg/ml.

As used herein the term 'a compound of Formula 1' means a compound being a furanone of the structure shown in Fig. 1, wherein R_1 is a hydrogen atom, a hydroxyl, ester or an ether group and wherein R_2 and R_3 are each a hydrogen atom or a halogen atom.

- 5 As used herein the term 'a compound of Formula 2' means a compound being a furanone of the structure shown in Fig. 2 wherein R_1 , R_2 , R_3 and R_4 are each a hydrogen, halogen, methyl, alkyl, hydroxyl, ether or ester group, or combinations of these groups.

- 10 The compounds used in the present invention consist of a series of structurally related furanones. Some of these compounds (Fig. 1) have been previously described in the literature, in part by the present inventors (Kazlauskas *et al.*, 1977; de Nys *et al.*, 1992; de Nys *et al.*, 1993), where their molecular structures have been elucidated and characterised by nuclear magnetic spectroscopy, mass spectrometer, ultra-violet and infra-red spectroscopy and optical rotation (α_D). The compounds all share a basic carbon skeleton consisting of a furanone moiety with an alkyl side chain at the 3-position of the basic structure. The substitution of hydrogen, hydroxy and acetoxy groups at the R_1 position, and hydrogen, bromine, chlorine and iodine at the R_2 , R_3 position is preferred and results in a large series of structurally related metabolites (Fig. 1). The combination of the most feasible substitutions at these positions results in 30 possible structures, of which 13 have been characterised. In addition, the present inventors have now tested several synthetic analogues of these compounds (Fig. 2 and 3), which, while sharing the basic furanone skeleton, differ in the length of their alkyl side chains. The present invention is intended to cover *inter alia*, the use of all of the 30 compounds in Fig. 1, as well as the extension of these structures shown in Figs 2 and 3.

- 30 The furanone compounds are close structural analogues of the AHL family of regulators (the structure of homoserine lactone and related regulatory molecules are described in Swift *et al.* 1994, and Fuqua *et al.* 1994), and it now seems that these compounds are in fact mimics of the homoserine lactone family of regulatory compounds. Thus the furanones are likely to be broadly useful as competitive inhibitors of these regulatory systems in a wide variety of applications.

In order that the present invention may be more clearly understood examples thereof are described with reference to the following drawings:-

Brief Description of the Drawings

- 5 Figure 1 shows the structure of furanones from *Delisea pulchra*, formula 1:
Figure 2 shows the structure of furanones from *Delisea pulchra*, formula 2:
Figure 3 shows the structure of furanones tested (compounds 1-5, 8, 10 and 12-16 in assays;
Figure 4 shows inhibition of AHL regulated swarming in *Serratia liquifaciens*
10 by furanones;
Figure 5 shows effect of furanone compound 4 on (a) swarming LB10 with 1.5% agar, (b) swimming LB10 with 0.3% agar, and (c) growth of *Proteus mirabilis* at 37°C; and
Figure 6 shows results of the effects of crude extracts of *Delisea* when coated
15 on plastic against (a) six strains of bacteria in the laboratory and (b) attachment of bacteria in the field.

Modes for Carrying out the Invention

METHODS

- For the tests described below, *Delisea pulchra* metabolites
20 (furanone compounds) were extracted as follows: The alga was collected from Cape Banks, NSW, Australia and frozen on collection. The tissue was subsequently freeze dried, extracted with dichloromethane, and the crude extract reduced *in vacuo*. Purified metabolites were isolated by vacuum liquid chromatography followed by high performance liquid chromatography
25 (de Nys *et al.*, 1993). The structural elucidation of purified metabolites was carried out using a combination of nuclear magnetic resonance spectroscopy and mass-spectrometry techniques. Previously characterised *Delisea* metabolites and synthetic analogues set out in Fig. 3 were used in biological testing.

- 30 The furanone compounds and their analogous were tested against microorganisms as outlined below. All tests (bioassays) were done in the laboratory under controlled conditions.

Inhibition of homoserine lactone mediated systems in bacteria: swarming, exoenzyme, indigo production, and bioluminescence

- 35 All bacteria were precultured in liquid LB medium or on LB plates at room temperature.

***Serratia liquifaciens* (swarming and exoprotease) assay**

Serratia liquifaciens was grown in liquid LB medium at room temperature to an optical density of 0.3 at 450nm and stable inoculated onto LB agar (0.7% agar) plates. Plates were incubated at 30°C and observed
5 regularly for swarming behaviour. The effect on swarming of *furanone* compounds was tested by adding (after sterilisation and cooling of the agar) compounds 1-13 (Fig. 3), in the concentration range of 5-100 µg/ml, to the plates.

For assays of production of exoproteases by *Serratia liquifaciens*
10 the bacteria were cultured as above and inoculated in liquid LB medium. The effects of *furanone* compounds were tested by adding compounds 3 and 4 (Fig. 3) at 100 µg/ml. Aliquots were withdrawn from the cultures, centrifuged and sterile filtered. Sixty microlitres of supernatant were added to wells in skim milk agar plates (1.5% skim milk in M9 medium). Plates
15 were incubated at room temperature and observed for clearing zones around the wells.

The effect on growth of *Serratia liquifaciens* by *furanone* compounds was also measured in liquid LB medium by comparing growth of cultures without the addition of compounds versus growth of cultures with
20 added *furanone* compounds (100 µg/ml of compounds 1-13 (Fig. 3) in separate cultures). Growth was measured as optical density at 450nm and followed over a 48hr period.

***Chromobacterium violencia* (indigo) assay**

A mutant of *Chromobacterium violencia* negative for the HSL
25 auto inducer was used in the assay. The strain was pour plated in LB medium at high density and wells were punched after the media had set. Sterile, filtered supernatants from various HSL producing strains were added to the wells. Plates were incubated at 30°C and observed for the formation of purple zones (due to indigo production) around the wells. The effects of
30 *furanone* compounds 1 and 2 (Fig. 3) were assessed by running this assay on plates of LB medium containing *furanone* compounds at a concentration of 100µg/ml. The effects of the compounds on growth of *Chromobacterium* were assessed as above.

***Pseudomonas aeruginosa* (elastase) assay**

Inoculants from precultures were streaked onto Peptone-Tryptic Soy Broth agar containing 0.3% elastin. Plates were incubated at 37°C and observed for hydrolysis of the elastin. The effect of furanone compounds was assessed by adding compounds 1-4 (100 µg/ml) to the plates and comparing zones of hydrolysis in plates with and without the compounds.

***Erwinia carotovora* (exoenzyme) assay**

Inoculants from precultured *E. carotovora* were added to slices of potatoes. Degradation (softening and browning) of the potato was compared on slices with and without furanone compounds (3 and 4).

***Vibrio fischeri* and *V. harveyi* (bioluminescence) assays**

Initial assays for both strains of *Vibrio* were streaked onto LB agar plates (with 2% NaCl) with and without furanone compounds 1-4 at 5-100 µg/ml. Bioluminescence of the plates was visually assessed after 24 hours of incubation at room temperature. In subsequent assays, both strains were grown in liquid culture in the presence of furanones. Bioluminescence was assayed via a luminometer. The effects of furanones on growth of both strains were assayed as above.

Inhibition of surface motility and adhesion

As well as specifically affecting well defined AHL mediated bacterial characteristics, furanones also inhibit other bacterial properties, in particular surface motility and adhesion. Like their interference with other AHL systems furanones interfere with or inhibit surface motility and swarming without necessarily having general growth inhibitory or microbiocidal effects. *Proteus mirabilis* and several characterised (but not yet taxonomically identified) marine isolates were used in these tests.

***Proteus mirabilis* and marine isolates (swarming assay)**

Methods followed those described for *Serratia liquifaciens* (above). Inhibition of growth of these bacteria by furanones was assessed as above.

Marine isolates (adhesion assays)

Six marine bacterial strains from submerged surfaces on the coast near Sydney were isolated and cultured as above in LB medium (with 2% NaCl). Attachment assays were done in two ways. Firstly, furanones were coated onto plastic dishes at several concentrations, and the dishes attached to frames. Each frame was placed in media which contained

10⁷/ml of one of the 6 bacterial isolates. After 2 hours, the frames were removed, rinsed thoroughly, stained with DAPI, and the number of attached bacteria on each dish via epifluorescence microscopy.

Secondly, inhibition of attachment was tested by pre-incubating the strains with furanones, so that the cells could take up the compounds. Three pre-incubation periods were used; 1/2, 3 and 10 hours. At the end of the pre-incubation period, the cells were washed, placed into experimental containers at 10⁷/ml, and plastic dishes (uncoated) placed into the containers. After 2 hours the frames with the dishes were removed, rinsed, and the number of attached cells counted as above.

Inhibition of growth of bacteria and fungi by compounds of Formula 2

In addition to the tests described above, the effects of selected compounds of the general formula 2 on growth of bacteria, one yeast (*Candida albicans*) and one fungus (*Helminthosporium sp.*) were assessed. Bacteria were grown in liquid media in the presence of different concentrations of furanones and growth measured via changes in optical density (OD). Fungal assays were done by inoculating agar plates with spores. These plates contained different concentrations of furanone compounds in wells within the plates. Zones of inhibition around these wells were measured after 48 hours.

RESULTS OF BIOASSAYS

Effects on AHL mediated processes

The effects of furanones on AHL dependent phenotypes, and growth, for the AHL assay strains number of bacterial species are shown in Table 1. In all instances some of the furanones affected AHL dependent phenotypes at concentrations that did not inhibit growth. The most complete set of results was obtained for *Serratia liquifaciens*, a facultative plant pathogen. None of the furanones tested inhibited growth or swimming behaviour of this organism. All compounds tested, however, inhibited swarming, a AHL dependent behaviour, to varying degrees (Fig 4a, 4b and Table 1). This demonstrates the presence of a mechanism that interferes with bacterial cell to cell communication and thereby limits bacterial surface colonisation without affecting growth or survival of the bacteria. In an analogous fashion, compounds 3 and 4 inhibit production of exoproteases by *Serratia exoproteases* at concentrations where growth is unaffected (none of the compounds tested inhibited growth of *Serratia liquifaciens* at 100 µg/ml).

Indigo formation in *Chromobacterium violencia*, an HSL regulated system, is inhibited by compounds 1 and 2 (Table 2). These two compounds have no effect on the growth of this bacterium. The indigo negative *Chromobacterium violencia* mutant did not produce the pigment in response to the addition of compounds 1 and 2 (e.g. the compounds do not stimulate indigo production). As for *Serratia*, the compounds appear to specifically interfere with an AHL mediated process, rather than having gross effects (e.g. inhibition of growth) on the organism. In both cases, the results suggest competitive inhibition of HSLs by the compounds of the invention. Growth of *Chromobacterium* was inhibited by 100 ug/ml of compound 4 but unaffected by the same concentration of compounds 1-3.

The compounds also affect AHL mediated processes in two important pathogens, the human pathogen *Pseudomonas aeruginosa* (cystic fibrosis) and the plant pathogen *Erwinia carotovora* (soft rot disease). Production of elastase, which is an AHL mediated virulence factor for *P. aeruginosa*, is stimulated by compounds 1 and 2, but inhibited by compounds 3 and 4. There is no detectable effect of compounds 2 and 4 on growth. The observation that structurally very similar compounds (1 and 2 versus 3 and 4) can have opposite effects on elastase production again indicates a very specific effect of the compounds on this HSL mediated system.

For *Erwinia carotovora*, compound 4 decreases exoenzyme production without inhibiting growth. Compound 3 has no effect on either growth or exoenzyme formation, again supportive of the specific effects of these metabolites.

Furanones inhibit AHL regulated bioluminescence in both *Vibrio fischeri* and *V. harveyi* without inhibiting growth of either strain (Table 2).

Table 1. Effect of furanone compounds on bacterial species with acylated homoserine lactone phenotypes

Organism	<i>Serratia liquefaciens</i>		<i>Chromobacterium violencia</i>	<i>Vibrio fischeri</i>	<i>Vibrio harveyi</i>
Significance	plant pathogen			marine symbiont	marine symbiont
HSL phenotype	swarming	exoenzymes	indigo formation	luminescence	luminescence
acyl-HSL type	HIB-HSL			OII-HSL a.o.	HB-HSL
Effect of	1	no effect	no effect	no effect	no effect
Furanone	2	no effect	no effect	no effect	no effect
compounds 1-4 on	3	no effect	slowed	no effect	no effect
bacterial growth	4	no effect	inhibit	no effect	no effect
Effect of	1	inhibit	inhibit	inhibit	inhibit
Furanone	2	inhibit	inhibit	inhibit	inhibit
compounds 1-4 on	3	inhibit	nt	inhibit	inhibit
HSL phenotype	4	inhibit	nt	inhibit	inhibit
Comments	* Growth and swimming is not inhibited by 100µg/ml of either compound. * 100 µg/ml of 3 and 4 decreases the exoprotease activity of the wild type and eliminates the exoprotease activity of the mutant		* Growth inhibited by 4 and slowed by 3 (each 100 µg/ml)	* All compounds inhibitory at 100 µg/ml. 4 most inhibitory <i>V. fischeri</i> more sensitive than <i>V. harveyi</i> * No visible effect on luminescence of lower concentrations (based on visual inspection of agar plates)	

HIB: N-(3-hydroxybutan-2-yl); OII: N-(3-oxohexan-2-yl); OOC: N-(3-oxooctan-2-yl); ODD: N-(3-oxododecan-2-yl) nt = not tested

Table 1. (continued)

Organism	<i>Pseudomonas aeruginosa</i>		<i>Erwinia carotovora</i>
Significance	opportunistic human pathogen cystic fibrosis		plant (potato) pathogen
HSL phenotype	elastase	Exotoxin A	exoenzymes
acyl-HSL type	ODD-HSL		OII-HSL
Effect of	1	nt	nt
Furanone	2	nt	nt
compounds 1-4 on	3	no effect	no effect
bacterial growth	4	no effect	no effect
Effect of	1	stimulated	nt
Furanone	2	stimulated	nt
compounds 1-4 on	3	delay/ inhibited	no effect
HSL phenotype	4	delay/ inhibited	inhibit
Comments	* Growth on plates unaffected by 100µg/ml of either compound * Elastase production is also highly media dependant		

Table 2. Minimum inhibitory concentrations of *Delisea pulchra* crude extract and furanone compounds inhibitory to four fouling relevant phenotypes: swimming, attachment, growth and swarming in strains isolated from *D. pulchra* and rock surfaces.

5

Strain	Compound	Concentration of <i>D. pulchra</i> crude extract (DC) or furanone compound (1-4) inhibiting four phenotypes			
		Swimming ($\mu\text{g/ml}$)	Attachment ($\mu\text{g/cm}^2$)	Growth(2) ($\mu\text{g/ml}$)	Swarming ($\mu\text{g/ml}$)
R12	DC	nd(1)	0.01	nd	nd
	D1	>50	nd	>50	5
	D2	>50	nd	>50	5
	D3	50	nd	>50	5
	D4	25	nd	25	5
R86	DC	nd	0.01	nd	nd
	D1	50	nd	>50	25
	D2	>50	nd	>50	5
	D3	>50	nd	>50	5
	D4	25	nd	25	5
R130	DC	nd	0.01	nd	nd
	D1	>50	nd	>50	25
	D2	>50	nd	>50	25
	D3	>50	nd	25	25
	D4	25	nd	25	5
V21	DC	nd	1.0	nd	nd
	D1	25	nd	25	5
	D2	25	nd	25	5
	D3	25	nd	5	5
	D4	5	nd	5	5
V54	DC	nd	1.0	nd	nd
	D1	5	nd	>50	25
	D2	5	nd	>50	5
	D3	5	nd	25	5
	D4	5	nd	25	5
V55	DC	nd	1.0	nd	nd
	D1	5	nd	>50	5
	D2	5	nd	>50	5
	D3	5	nd	5	5
	D4	5	nd	25	5

(1) not done

(2) in liquid media

Inhibition of surface motility and adhesion

Both crude extract of *Delisea*, and compound 4, inhibited swarming by the opportunistic human pathogen *Proteus mirabilis*.

Compound 4 did not affect growth or swimming at concentrations that were inhibitory to swarming (Fig 5). Swarming of all 6 marine strains was also inhibited by furanones at concentrations that did not inhibit growth (Table 2).

When the crude non-polar extract of *Delisea* was coated onto artificial surfaces in laboratory assays it inhibited adhesion by all six marine isolates tested. Inhibition of some strains occurred at concentrations as low as 10 ng/cm²; 5 of 6 strains were inhibited by 1 ug/cm² (Fig 6A). Results of field assays of bacterial adhesion were consistent with these results. Crude *Delisea* extract coated onto surfaces at 1 ug/cm² decreased bacterial adhesion by >90% in the field (Fig 6B).

Pre-incubation of bacteria with crude non-polar extract of *Delisea*, followed by washing of the cells, also significantly inhibited subsequent adhesion of the bacteria to (uncoated) inanimate surfaces. This result is particularly telling with regard to the results for inhibition of AHL regulatory systems described above. This is because the pre-incubation experiments demonstrate that the furanones are not simply inhibiting adhesion by changing surface characteristics. Rather they are taken up by the cells, with the result that adhesion is then inhibited, and indicating that a regulatory system has been affected resulting in a loss of production of adhesin[s]. Further evidence that the regulatory system affected is AHL driven comes from the observation (data not shown) that when cells are pre-incubated with AHLs, adhesion increases. Although many adhesion assays were done with crude extract of *Delisea*, it will be appreciated by one skilled in the art that the effects are due to the furanones which are contained within the extract.

Inhibition of fungal growth by compounds of Formula 2

All compounds inhibited either *Candida albicans* or *Helminthosporium* sp. for at least one of the levels tested (Table 3). The compounds were most effective against *Helminthosporium*, with activities in some cases comparable to that of the commercial fungicide Amphotericin B. At the highest levels tested, growth of *Helminthosporium* was completely inhibited by 4 of the furanones: unlike the effects of Amphotericin B. It is

also emphasised that these assays underestimate the effects of these compounds relative to the commercial product, since diffusion of these compounds through agar is much less than that of the water soluble amphotericin B.

5 These results clearly demonstrate that natural furanones and synthetic analogues:

- 10 (i) Specifically interfere with bacterial properties that are regulated by homoserine lactone or acylated homoserine lactone regulatory systems. Specific properties known to be regulated by AHLs shown to be affected here include swarming, exoenzyme production, bioluminescence, and pigment production. Adhesion, which is stimulated by the addition of AHLs and is also likely to be AHL regulated, was also inhibited by the furanones tested. Similarly, swarming of *Proteus mirabilis* and a number
- 15 marine isolates, was also inhibited by the furanones. It should be noted that other AHL regulated processes or properties not tested here are also likely to be inhibited by furanones including virulence in the fish pathogen *Vibrio anguillarum*, plasmid transfer in *Agrobacterium tumefaciens*.
- 20 (ii) Some of the compounds (formula 2) tested also inhibit growth and or kill both bacteria and fungi.

Table 3 Inhibition of fungal growth by furanones. Data are width (mm) of inhibition zone.

Candida albicans

Compound	Amount Added (μ g)			
	100	10	1	0.1
Control		no inhibition		
Ethanol		no inhibition		
Amphotericin B	1-14	6-10	5-7	2-4
Compound 1	1-2	0	0	0
Compound 2	2-3	0	0	0
Compound 3	2-3	1-2	0	0
Compound 5	3-8	1	0	0
Compound 8	0	0	0	0
Compound 13	1	0	0	0

Helminthosporium sp

Compound	Amount Added (μ g)			
	100	10	1	0.1
Control		no inhibition		
Ethanol		no inhibition		
Amphotericin B	7-10	6-8	6	2
Compound 1	20	2-5	0	0
Compound 2	20	2-4	0	0
Compound 3	6-20	6-9	0	0
Compound 5	20	9-13	2-4	0
Compound 8	20	0	0	0
Compound 13	0	0	0	0

Thus these compounds, taken as a whole, can interfere with AHL driven systems in microorganisms as well as inhibit growth of some strains. The effects are highly specific and are dependent on concentration of the furanone compounds, on the strain tested and on the structure of the furanones and on the given AHL mediated system. Furanones can be used to specifically inhibit HSL systems without inhibiting growth, as well as being used to inhibit growth. Because of the widespread occurrence of HSL mediated functions in bacteria, furanones in this context may be useful in many applications, including but not restricted to biomedical, agricultural, and antifouling applications

It is noted that some previous work on the activity of some of these compounds as biocidal agents has been done, in particular the results reported by Reichelt and Borowitzka (1984). Their results show that several of the furanones of Formula 1 inhibited growth of bacteria, however, there is no indication or teaching that furanones can effect specific regulatory systems. The present inventors have found that furanones strongly inhibit specific properties (exoenzyme production, swarming, etc.) of these bacteria and these compounds can be used in a variety of *in vitro* and *in vivo* systems to effect such properties.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all aspects as illustrative and not restrictive.

References

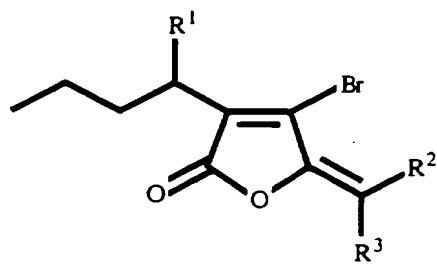
- de Nys, R., Coll, J.C., Bowden, B.F. (1992). *Delisea pulchra* (cf. *fimbriata*) revisited. The structural determination of two new metabolites from the red alga *Delisea pulchra*. *Aus. J. Chem.* 45: 1625-1632.
- 5 de Nys, R., Wright, A.D., Konig, G.M., Sticher, O. (1993). New halogenated furanones from the marine alga *Delisea pulchra* (cf. *fimbriata*). *Tetrahedron* 49: 11213-11220.
- Fletcher, R.L. (1989). A bioassay technique using the marine fouling green alga *Enteromorpha*. *Int. Biodeterioration* 25: 407-422.
- 10 Fuqua, W.C., Winans, S.C., Greenberg, E.P. (1991) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriology* 176: 269-275.
- Jefford, C.W., Jaggi, D., Boukouvalas, J. (1988). *J. Chem. Soc., Chem. Comm.* : 1595.
- 15 Jefford, C.W., Jaggi, D., Sledeski, A.W., Boukouvalas, J. (1989) *Stud. Nat. Prod. Chem.*, 3: 157.
- Kazlauskas, R., Murphy, P.T., Quinn, R.J., Wells, R.J. (1977). A new class of halogenated lactones from the red alga *Delisea fimbriata* (*Bonnemaisoniaceae*). *Tet. Lett.* 1: 37-40.
- 20 Reichelt, J.L., Borowitzka, M.A. (1984) Antimicrobial activity from marine algae; results of a large scale screening programme. *Hydrobiologia* 116/117:158-168.
- Swift, S., Bainton, N.J., Winson, M.K. (1994). Gram-negative bacterial communication by N-acyl homoserine lactones: a universal language?
- 25 *Trends in Microbiology* 2: 193-198.

Claims

1. A method of inhibiting an homoserine lactone (HSL) and/or acylated homoserine lactone (AHL) regulated process in a microorganism comprising exposing the microorganism to a furanone compound so as to inhibit the process.
2. The method according to claim 1 wherein the furanone compound is derived from the alga *Delisea pulchra* or chemical modifications or derivatives thereof.
3. The method according to claim 2 wherein the furanone compound is selected from the group consisting of the compounds of Formula 1 and Formula 2 as hereinbefore defined.
4. The method according to claim 3 wherein the furanone compound is selected from the group consisting of the compounds of Figure 3.
5. The method according to any one of claims 1 to 4 wherein the regulated process is selected from the group consisting of motility, swarming, swimming, exoenzyme production, indigo formation, luminescence, adhesion and attachment.
6. The method according to any one of claims 1 to 5 wherein the furanone compound is used at a concentration greater than 10 ng/ml.
7. The method according to claim 6 wherein the furanone compound is used at a concentration from 100 ng to 1 mg/ml.
8. The method according to claim 7 wherein the furanone compound is used at a concentration from 1 µg to 100 µg/ml.
9. The method according to claim 5 wherein the regulated process is motility or swarming and the furanone compound is incorporated in a microbial culture medium at a concentration greater than 10 ng/ml so as to substantially inhibit motility or swarming of the microorganism.
10. The method according to claim 9 wherein the furanone compound is incorporated in the culture medium at a concentration from 1 µg to 100 µg/ml.
11. The method according to claim 5 wherein the regulated process is adhesion or attachment and the furanone compound is applied to a surface at a concentration greater than 1 ng/cm² so as to substantially inhibit adhesion or attachment of the microorganism to the surface.

12. The method according to claim 11 wherein the furanone compound is applied to the surface at a concentration from 10 ng to 10 $\mu\text{g}/\text{cm}^2$.
13. The method according to any one of claims 1 to 12 wherein the
5 microorganism is a pathogen of plant, crustacean, fish, animal or human.
14. The method according to claim 13 wherein the plant pathogen is selected from the group consisting of *Serratia liquiaciens* and *Erwinia carotovora*.
15. The method according to claim 13 wherein the crustacean
10 pathogen is *Vibrio harveyi*.
16. The method according to claim 13 wherein the fish pathogen is *Vibrio anguillarum*.
17. The method according to claim 13 wherein the human pathogen is selected from the group consisting of *Pseudomonas aeruginosa* and *Proteus*
15 *mirabilis*.
18. A microbial culture medium for inhibiting an homoserine lactone (HSL) and/or acylated homoserine lactone (AHL) regulated process in a microorganism, the medium comprising growth and/or maintenance ingredients and a furanone compound.
- 20 19. The medium according to claim 18 wherein the furanone compound is derived from the alga *Delisea pulchra* or chemical modifications or derivatives thereof.
20. The medium according to claim 19 wherein the furanone compound is selected from the group consisting of the compounds of
25 Formula 1 and Formula 2 as hereinbefore defined.
21. The medium according to claim 20 wherein the furanone compound is selected from the group consisting of the compounds of Figure 3.
22. The medium according to any one of claims 18 to 21 wherein
30 the furanone compound is included in the medium to give a final concentration in use of greater than 10 ng/ml.
23. The medium according to claim 22 wherein the furanone compound has a final concentration from 100 ng to 1 mg/ml.
24. The medium according to claim 23 wherein the furanone
35 compound has a final concentration from 1 μg to 100 $\mu\text{g}/\text{ml}$.

1/6



<u>R1</u>	<u>R2</u>	<u>R3</u>
H	Br	Br
H	H	Br
H	Br	H
H	H	Cl
H	Cl	H
H	H	I
H	I	H
H	Cl	Cl
H	I	I
H	H	H
OH	Br	Br
OH	H	Br
OH	Br	H
OH	H	Cl
OH	Cl	H
OH	H	I
OH	I	H
OH	Cl	Cl
OH	I	I
OH	H	H
OAc	Br	Br
OAc	H	Br
OAc	Br	H
OAc	H	Cl
OAc	Cl	H
OAc	H	I
OAc	I	H
OAc	Cl	Cl
OAc	I	I
OAc	H	H

Fig. 1

2/6

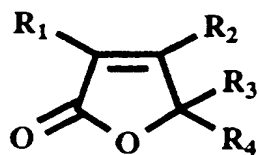
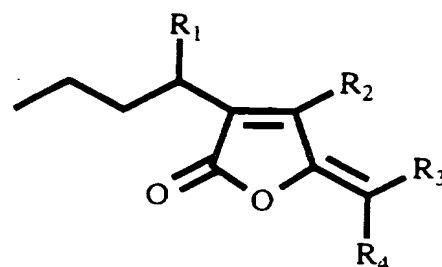


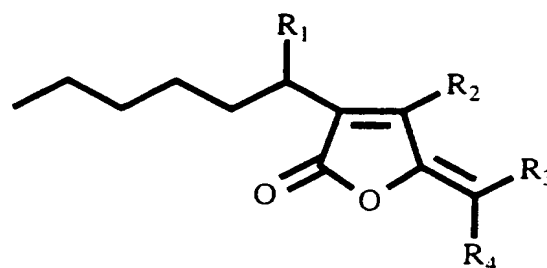
Fig. 2

3/6

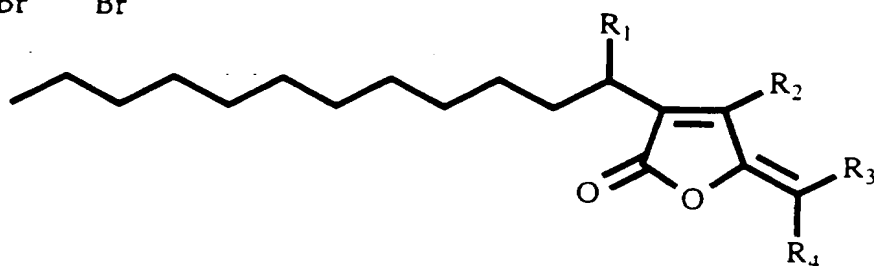
	<u>R1</u>	<u>R2</u>	<u>R3</u>	<u>R4</u>
1	H	Br	Br	Br
2	H	Br	H	Br
3	OAc	Br	H	Br
4	OH	Br	H	Br
5	OAc	Br	H	I
8	H	H	Br	Br
10	OAc	Br	Br	Br



	<u>R1</u>	<u>R2</u>	<u>R3</u>	<u>R4</u>
12	H	Br	H	Br
13	H	H	Br	Br



	<u>R1</u>	<u>R2</u>	<u>R3</u>	<u>R4</u>
14	H	H	Br	Br



	<u>R1</u>	<u>R2</u>	<u>R3</u>	<u>R4</u>
15	H	Br	Br	Br
16	H	Br	H	Br
17	H	H	Br	Br

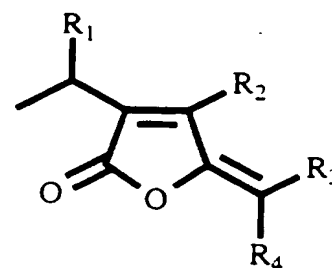


Fig. 3

4/6

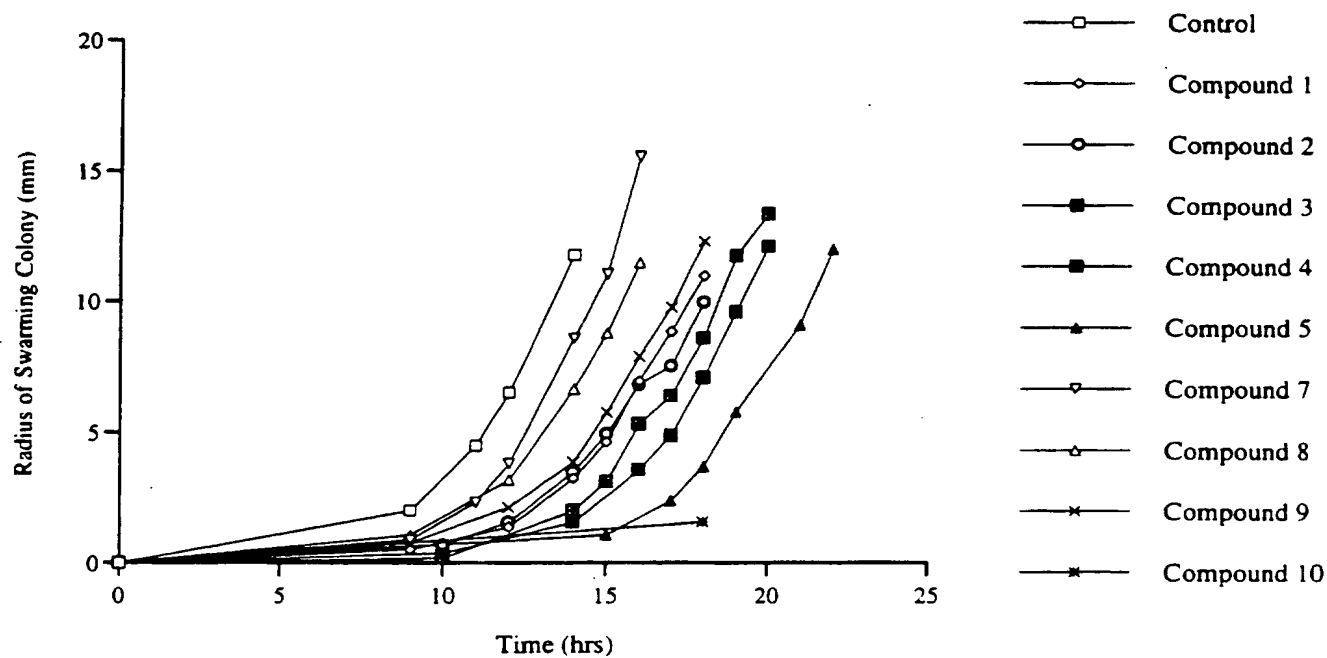


Fig. 4a

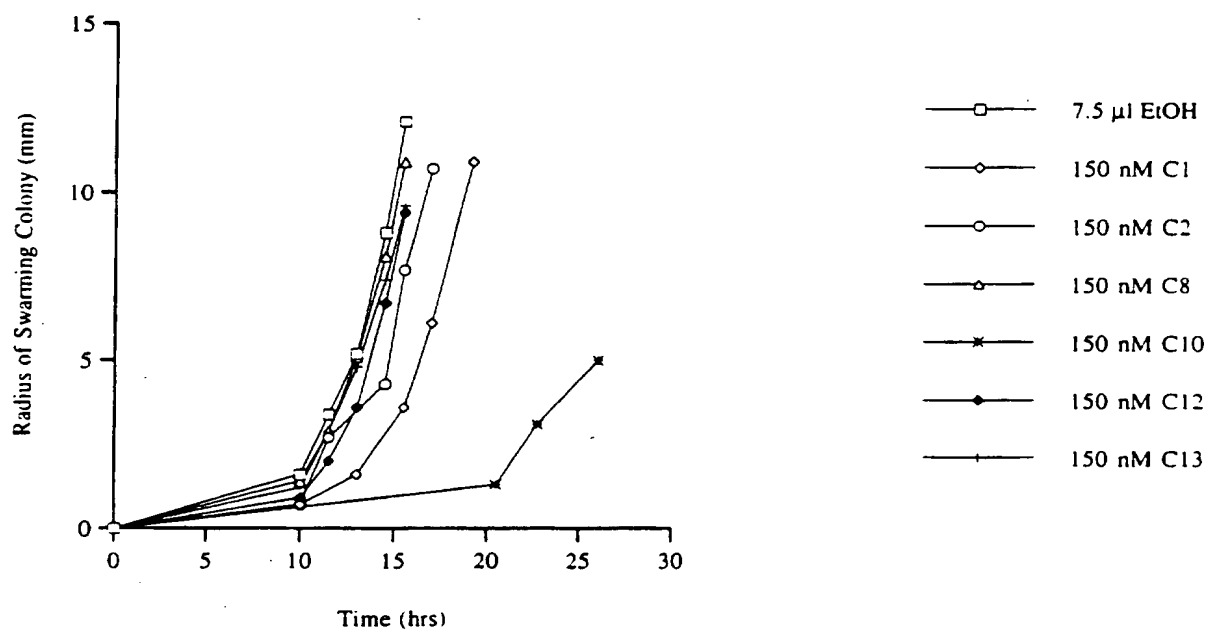
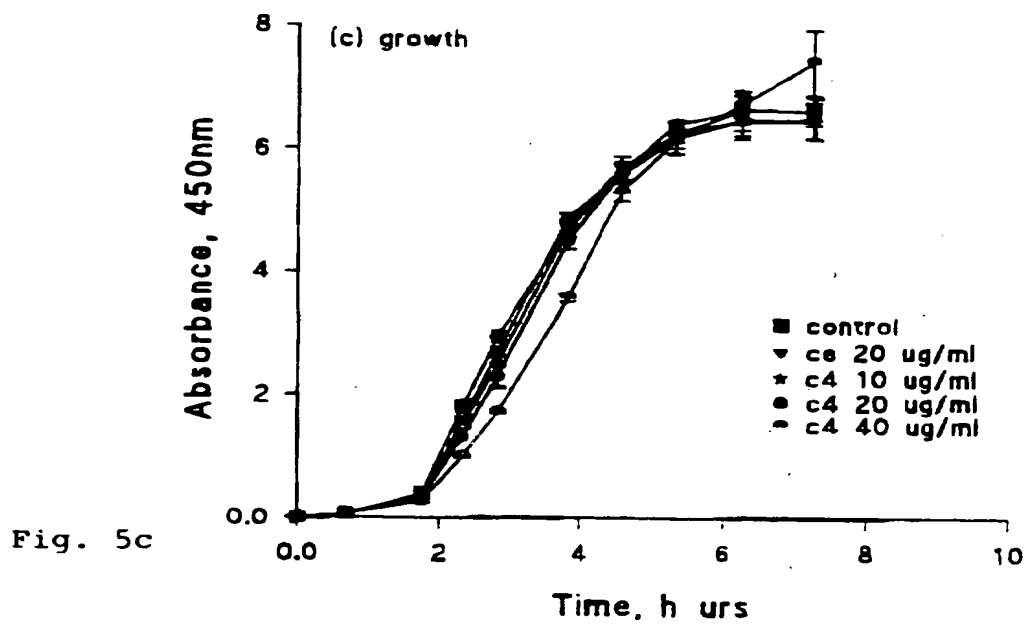
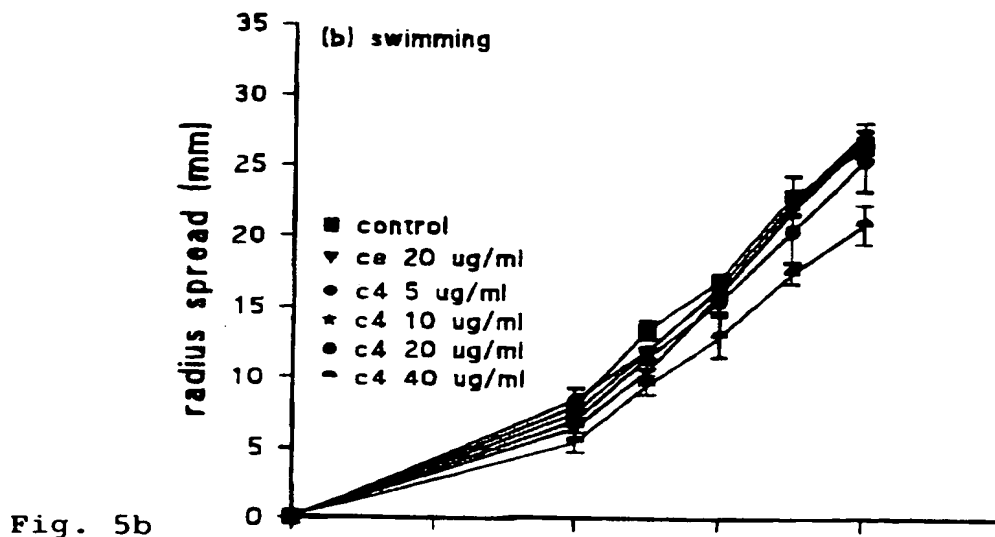
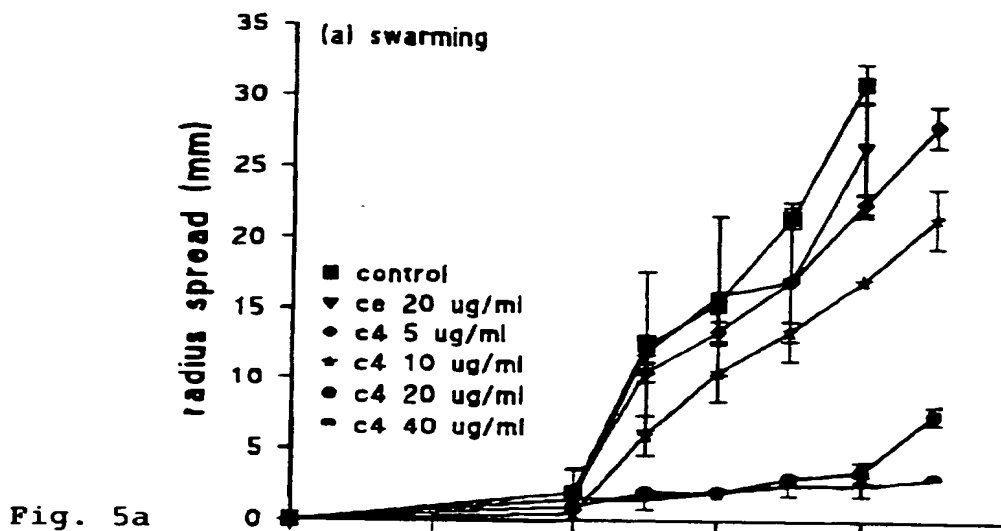


Fig. 4b

5/6



6/6

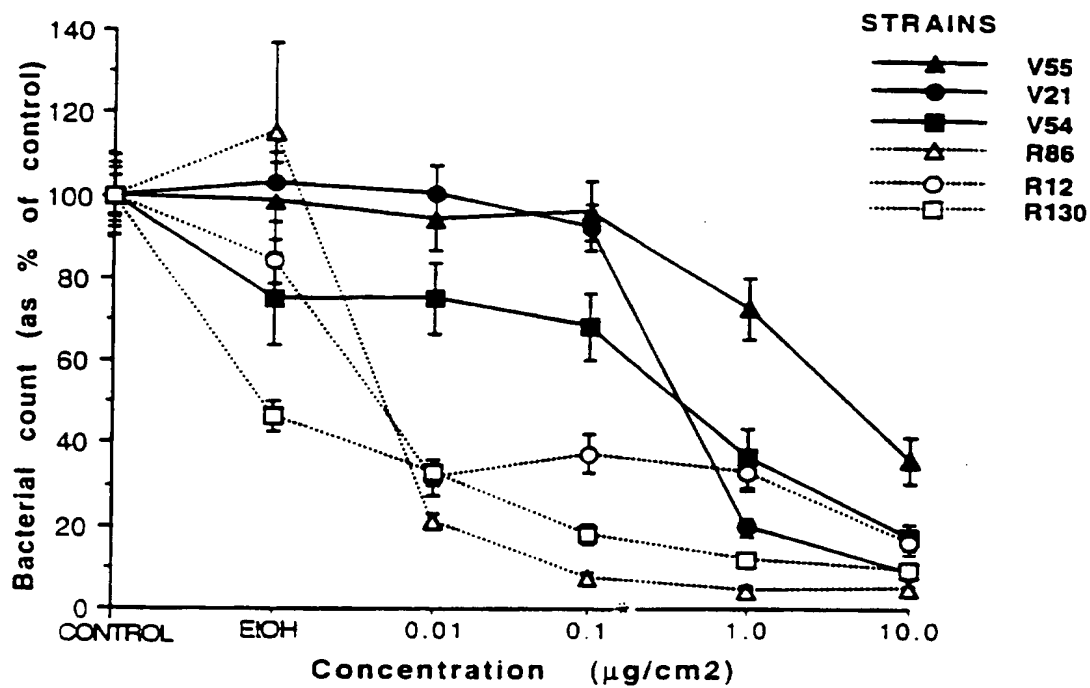


Fig. 6a

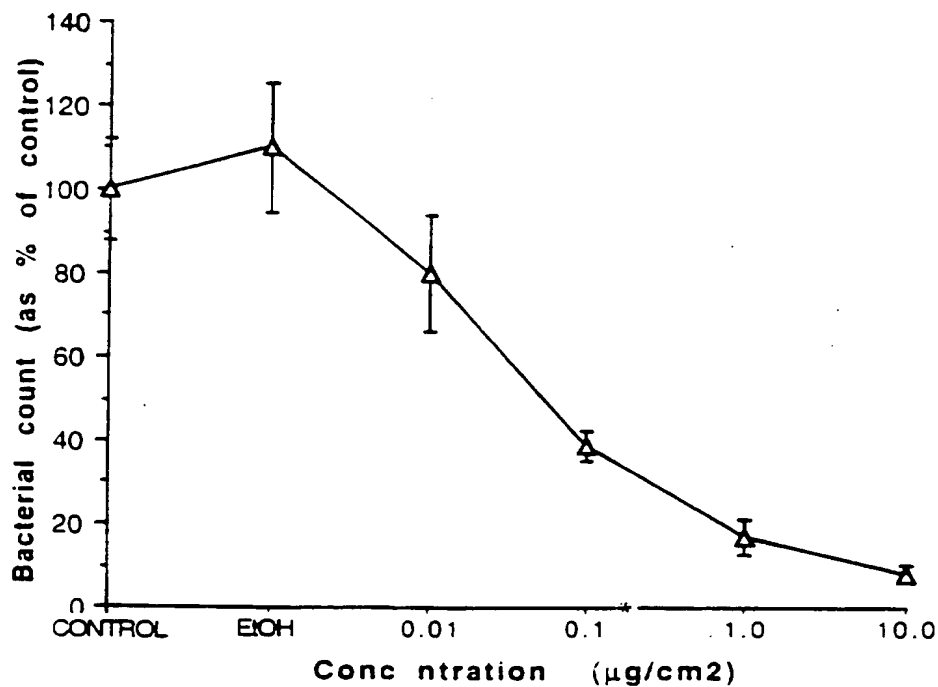


Fig. 6b

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00167

A. CLASSIFICATION OF SUBJECT MATTER		
Int Cl ⁶ : C12N 1/20, 9/99		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC: C12N; C12P; C12Q; C07K; C61K; Chemical Abstracts; All through electronic databases		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
WPAT & JAPIO Keywords: Please see supplemental box		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J L REICHELT & M A BOROWITZKA: "Anumicrobial activity from marine algae: Results of a large-scale screening programme". Hydrobiologia, volume 116/117, pages 158-168, 1984 See whole document	1-24
A	US 5196318 (T O BALDWIN et al.), 23 March 1993, IPC ⁵ C12P, C12N See whole document	1-24
A	G W HUISMAN & R KOLTER: "Sensing Starvation: A Homoserine Lactone-Dependent Signaling Pathway in <i>Escherichia coli</i> " Science, volume 265, pages 537-539, 22 July 1994 See whole document	1-24
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 6 May 1996		Date of mailing of the international search report 13TH MAY 1996.
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929		Authorized officer ROBYN PORTER Telephone No.: (06) 283 2318

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 96/00167

Box V

WPAT & JAPIO Keywords:

ss1a: homo(w)serine or homoserine

ss2a: 1(5N)Lactone #

ss1b: Lux#

ss2b: Regulat: or control: or inhibit:

ss3b: 1 and 2

ss4b: 3 and (C12N/IC or C12P/IC or C12Q/IC or C07K/IC or A61K/IC or G01N-033/IC)

ss1c: Homo(w)serine or homoserine

ss2c: 1(5N)Lactone#

ss3c: Inhibit: or control: or regulat:

ss4c: 2(10N) 3

Chemical Abstracts Keywords

ss1: (?Homoserine)(3N)Lactone#/IT

ss2: Lux#/IT

ss3: (Regulat? or control? or inhibit?)/IT

ss4: 2 or 3

ss5: 4 and 1

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 96/00167

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member
US	5196318	NONE

END OF ANNEX



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 1/20, 9/99	A1	(11) International Publication Number: WO 96/29392 (43) International Publication Date: 26 September 1996 (26.09.96)
(21) International Application Number: PCT/AU96/00167 (22) International Filing Date: 25 March 1996 (25.03.96) (30) Priority Data: PN 1912 23 March 1995 (23.03.95) AU (71) Applicant (for all designated States except US): UNISEARCH LIMITED [AU/AU]; 221-227 Anzac Parade, Kensington, NSW 2033 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only): KJELLEBERG, Staffan [SE/AU]; 22 Goorawahl Avenue, La Perouse, NSW 2036 (AU). STEINBERG, Peter [US/AU]; 144 Denison Street, Newtown, NSW 2042 (AU). DE NYS, Peter, Canisius [NZ/AU]; 3/7 Palmerston Avenue, Bronte, NSW 2024 (AU). MAXIMILIEN, Ria [AU/AU]; 138 Kurrewa Avenue, Coogee, NSW 2034 (AU). MANEFIELD, Michael [AU/AU]; 2/40 Wairoa Avenue, Bondi Beach, NSW 2030 (AU). GIVSKOV, Michael [DK/DK]; Soetedams Dosseringen 59C, DK-2100 Copenhagen (DK). GRAM, Lone [DK/DK]; Ellemosevej 20, DK-2900 Hellerup (DK). (74) Agent: F. B. RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: METHODS FOR MICROBIAL REGULATION (57) Abstract A method and microbial culture medium for inhibiting homoserine lactone (HSL) and/or acylated homoserine lactose (AHL) regulated processes in microorganisms using furanone compounds.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam